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ORIGINAL ARTICLE

Molecular identification and phylogenetic relationship of green algae, *Spirogyra ellipsospora* (Chlorophyta) using ISSR and rbcL markers

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KEYWORDS

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Abstract *Spirogyra* is found in a wide range of habitats, including small stagnant water bodies, rivers, and streams. *Spirogyra ellipsospora* is common in northern Thailand. Species identification of the *Spirogyra* species based only on morphological characteristics can be difficult. A reliable and accurate method is required to evaluate genetic variations. This study aims to apply molecular approaches for the identification of *S. ellipsospora* using microsatellites and rbcL markers. Based on DNA sequencing, the rbcL gene was sequenced and the data was analyzed using the BLAST (Basic Local Alignment Search Tool) program in the NCBI (National Center for Biotechnology Information) database. The sequence of *S. ellipsospora* from this study revealed definitive identity matches in the range of 99% for the consensus sequences of *S. ellipsospora*. The 10 primers of ISSR could be amplified by 92 amplification fragments. The DNA fragments and the rbcL sequence data grouped the *Spirogyra* specimens into two distinct clusters.

1. Introduction

Spirogyra is a genus of filamentous green algae in the order Zygnematales. The name indicates the helical or spiral arrangement of the chloroplasts, which is the main diagnostic characteristic of the genus. The *Spirogyra* species typically develops unbranched filaments and is one cell thick, which grows longer through normal cell division. There are more than 400 species of *Spirogyra* in the world. Identification of a particular *Spirogyra* species is accomplished by microscopic examination of the spores (Thiamdao and Peerapornpisal, 2011).

Vegetative growth of *Spirogyra* can be recognized by three characteristics: (i) type of cross walls (plane, replicate, semi-replicate or colligate), (ii) cell length and width and (iii) chloroplast numbers. The process of conjugation can be included for species identification (Berry and Lembi, 2000; Hainz et al., 2009). A morphological examination of *Spirogyra* reveals that it has spiral chloroplasts, pyrenoids, and a nucleus.

The morphology of some species of *Spirogyra* and *Cladophora* shows similar cell shapes and spiral chloroplasts. There have been few reports published on the diversity of *Spirogyra*

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in Thailand. Thiamdao and Peerapornpisal (2011) have previously investigated the morphology of *Spirogyra ellipsospora* in northern Thailand. This species is generally eaten raw in the north and northeast regions of Thailand. It goes by the local name tao, thao, or phakkai. Moreover, it is considered cosmopolitan and thrives in a wide range of habitats, including small stagnant water bodies and ditches, as well as in the littorals of lakes, rivers and streams. Cobble and gravel substrates are the preferred habitats of macroalgae. *Spirogyra* has been found to be in greater abundance during the hot, dry season and before the rainy season (Hainz et al., 2009).

However, the identification of *S. ellipsospora* has mainly been based on conjugation and zygospores, but is mostly found in the vegetative stage. Species identification of related *Spirogyra* species based on morphological characteristics can be difficult. Moreover, difficulties arise because this species is small and soft and also has only a few stable morphological characteristics and is subject to phenotypic variations. Thus, the identification of closely related species of *Spirogyra* has only been based on morphological characteristics and thus, it can be confused or misidentified. Molecular methods are useful in an evaluation of the genetic variations, as well as for accurate identification.

Molecular procedures using the PCR technique have been applied to support taxonomic evidence that is related to certain diverse organisms including algae. The microsatellite markers (ISSR) (Widmer et al., 2010) have been applied widely in the species identification of many living organisms, including fungus (Lihme et al., 2009; Alaniz et al., 2009), fruit plants (Hussein et al., 2008), beans (Galván et al., 2003), and green algae (Shen, 2008). This technique has been reported to be highly reproducible and to have a high level of specificity. ISSR markers have great potential and benefit in terms of studying genetic variations, phylogeny, gene tagging, genome mapping, and evolutionary biology. In addition, the ISSR PCR method has been reported to produce more complex marker patterns than the RAPD approach (Parsons et al., 1997), which is advantageous when differentiating between closely related cultivars. Moreover, the ISSR PCR approach is more reproducible than RAPD RCR, because the ISSR primers were designed to anneal to a microsatellite sequence. The ISSR PCR approach is more stable than the RAPD approach due to the fact that the primers for ISSR PCR are usually longer (16–20 bp) than those for RAPD (10 bp), which allows for a higher stringent condition. The ISSR approach has been proven to be more reliable than RAPD, because the primers of ISSR repeat sequences, can mutate more quickly than in the encoding region. If any differences appear in the genomes of the two species, they would be presented in polymorphic bands. Hence, the ISSR markers have been applied in many research studies and it is clear that the ISSR markers have great potential and could be highly beneficial in studying genetic variations, phylogeny, gene tagging, genome mapping and evolutionary biology (Wolff and Morgan-Richards, 1998; Reddy et al., 2002).

The ribulosebiphosphate carboxylase (rbcL) sequence method has been extensively used in studies of evolution, phylogeny, biogeography, population genetics, and systematics because it can be readily copied and not strikingly different for related species (Sheng-Guo et al., 2008; Doyle et al., 1997). The sequence of rbcL has been recorded in many studies and it is clear that this marker has great potential and benefit in terms of studying the genetic variations of the natural populations (Hamdam et al., 2013). This gene is far more variable in sequence. Because of the relatively rapid rate at which new mutants are fixed, these regions may be distinguished closely with other related species that otherwise would show little genetic divergence (Hamdam et al., 2013).

Our study aimed to determine the molecular identification, genetic relationships, and development of DNA markers of *S. ellipsospora*, using microsatellite markers and rbcL sequencing.

2. Materials and methods

2.1. *Spirogyra* specimens

S. ellipsospora was collected from the Chiang Mai Province, Thailand. While *Spirogyra* sp.1, *Spirogyra* sp.2, *Spirogyra* sp.3, and *Spirogyra* sp.4 were collected from the Nakron Sawan, Nan, Loei, and Saraburi Provinces, Thailand, respectively. Fresh specimens were examined as wet mounts under a light microscope and were then visualized using an Olympus DP 20 Model visualizer. The length, width, number of spiral chloroplasts, and number of granules were recorded for species confirmation.

2.2. Total genomic DNA of Spirogyra extraction

Total genomic DNA of all *Spirogyra* specimens was extracted and purified using the modified plant tissue extraction protocol (Dellaporta et al., 1983). Analysis of DNA quality and quantity was performed by 1.4% gel electrophoresis and optical density using a spectrophotometer at 260 and 280 nm, respectively.

2.3. Issr-pcr

Total genomic DNA of *Spirogyra* specimens was recorded by the Inter simple sequence repeat (ISSR) PCR technique. Ten ISSR primers were used individually for ISSR-PCR (Table 1). PCR conditions were used as follows: 1 cycle of 94 °C for 5 min, 40 cycles of 94 °C for 20 s, 51 °C for 1 min, 72 °C for 20 s and 1 cycle of final extension at 72 °C for 6 min, respectively.

2.4. Amplification of the rbcL gene

Analysis of Polymerase Chain Reaction (PCR) was carried out using rbcL-F (ATGTCA CCACAAACAGAGACTAAAGC) and rbcL-R (GTAAAATCAAGTCCACCRCG) primers. PCR conditions were as follows; 1 cycle of 94 °C for 4 min, 30 cycles of 94 °C for 1 min, 55 °C for 30 s, 72 °C for 45 s and 1 cycle of final extension at 72 °C for 7 min. The 1.4% agarose gel electrophoresis with ethidium bromide staining was used to visualize rbcL PCR products. The sequences were performed in order for the products to be checked by the BLAST program in the NCBI (National Center for Biotechnology Information) database, to confirm the PCR target. The electropherograms of each sequence were examined for sequence accuracy using a Sequence Scanner version 1.0 and Bioedit version 7.1. All sequences were aligned automatically using Clustal X version 2.0.

Table 1 Ten ISSR primers used to generate DNA fragment by PCR reactions.

Primer	Sequence 5′ → 3′	Length
UBC 807	AGA GAG AGA GAG AGA GT	17
UBC 808	AGA GAG AGA GAG AGA GC	17
UBC 809	AGA GAG AGA GAG AGA GG	17
UBC 825	ACA CAC ACA CAC ACA CT	17
UBC 826	ACA CAC ACA CAC ACA CC	17
UBC 827	ACA CAC ACA CAC ACA CG	17
UBC835	AGA GAG AGA GAG AGA GYC	18
UBC 857	ACA CAC ACA CAC ACA CYG	18
UBC864	ATG ATG ATG ATG ATG ATG	18
UBC880	GGA GAG GAG AGG AGA	18

2.5. Phylogenetic analysis

The rbcL sequences of all *Spirogyra* samples were determined by direct sequencing. Phylogenetic relationships among *Spirogyra* specimens were analyzed based on rbcL sequence data using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in the MEGA program (version 5.0). Seven isolates of *Spirogyra* from the Genbank database were used to construct the phylogenetic tree (Table 2) (see Table 3).

3. Results

The general morphology of *Spirogyra* is characterized by a coiled chloroplast and a light green color. The cell is cylindrical. Apical cells are tapering, with rounded tips and thick cell walls. There are five different morphological triads of the *Spirogyra* specimens. The arrangement of chloroplast spirals and granules of patterns 1 and 5 was highly condensed and compacted, while patterns 2, 3 and 4 were relatively scattered, as indicated (Triads 1): condensed and slightly compacted chloroplast spiral, (Triads 2): short cell with scattered chloroplast spiral, (Triads 3): long cell with less chloroplast spiral, (Triads 4): short cell with less chloroplast spiral and (Triads 5): long cell with condensed and compacted chloroplast spiral (see Fig. 1).

Table 2 List of materials and sequences of rbcL used for constructed phylogenetic analysis.

Species of Spirogyra	References
S. ellipsospora	This study
S. neglecta	This study
Spirogyra Sp.1	This study
Spirogyra Sp.2	This study
Spirogyra Sp.2	This study
S. ellipsospora	DQ 995996
S. ellipsospora	DQ995997
Spirogyra Sp.	KC779222
Spirogyra Sp.	KC779220
Spirogyra Sp.	KC779219
S. maxima	KC779213
S. maxima	KC779217

In terms of the molecular investigation, ninety-two scorable markers were produced using ten ISSR primers. The cluster analysis of the ISSR markers separated *S. ellipsospora*, other *Spirogyra* species and *Cladophora* sp. as out-groups into two district clusters, which included (cluster 1): *S. ellipsospora*, *Spirogyra* sp.1, and *Spirogyra* sp.4 and (cluster 2): *Spirogyra* sp.3, *Spirogyra* sp.2, and *Cladophora* sp. (Fig. 2).

Nucleotide amplification of rbcL revealed about 570 bp fragments in each *Spirogyra* specimen. Based on the rbcL sequencing data we obtained, they were trimmed to provide an equivalence sequence among each morphological triad. The specific DNA fragment of rbcL was analyzed using the BLAST (Basic Local Alignment Search Tool) program in the NCBI (National Center for Biotechnology Information) database. Sequence data of *S. ellipsospora* from our study revealed definitive identity matches for *S. ellipsospora* for consensus sequences with 2 accession numbers of *S. ellipsospora* that are available on the NCBI database.

Phylogenetic trees were analyzed for the rbcL sequences using UPGMA. The phylogram could be separated into two district clusters (cluster 1): *S. ellipsospora*, *Spirogyra* sp.2, and *Spirogyra maxima* and (cluster 2): *Spirogyra* sp.1, *Spirogyra* sp.3, *Spirogyra* sp.4, and *Spirogyra* sp. (Fig. 3).

4. Discussion

At present, classical morphologically based methods and molecularly based methods are used for the identification of *Spirogyra* specimens, which are wildly distributed throughout all parts of Thailand. However, the phenotypic traits may lead to misidentifications and they may be more sensitive than with the molecular identification approach. The *Spirogyra* specimens were collected and then classified into five patterns under a light microscope.

Table 3 Morphological characteristic of each *Spirogyra* specimens.

Details	*S. ellipsospora*	Sp.1	Sp.2	Sp.3	Sp.4
Vegetative cell width (μm)	45–90	40–55	40–60	41–50	40–60
Vegetative cell length (μm)	110–225	85–180	95–188	122–161	95–188
L/W ratio vegetative cell	2.4–2.5	2.13–3.27	2.38–3.13	2.97–3.22	2.35–3.13
Number of chloroplasts	2–3	2	3	2	4–5
Shape of zoospore	Ellipsoid	ns	ns	ns	ns
Zoospore width	55–70	ns	ns	ns	ns
Zoospore length	80–90	ns	ns	ns	ns
L/W ratio zoospore	1.3–1.4	ns	ns	ns	ns
Shape of pyrenoid	Discoid				

Remark: ns = not seen.

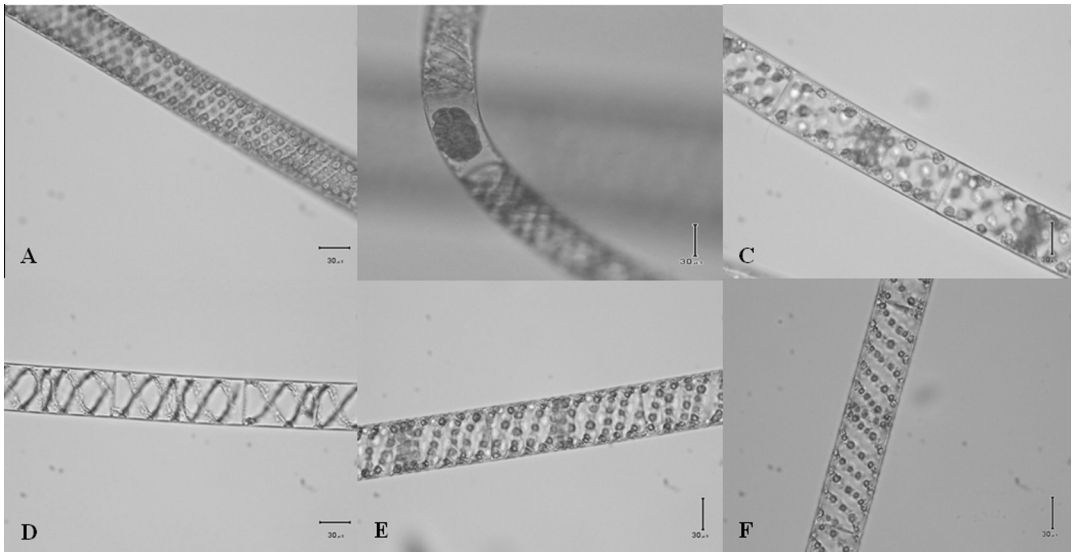

Figure 1 Five different morphological triads of *Spirogyra* specimens. (A) *Spirogyra ellipsospora*, (B) zoospore of *S. ellipsospora*, (C) *Spirogyra* sp.1, (D) *Spirogyra* sp.2, (E) *Spirogyra* sp.3, (F) *Spirogyra* sp.4 (scale bar = 30 μm).

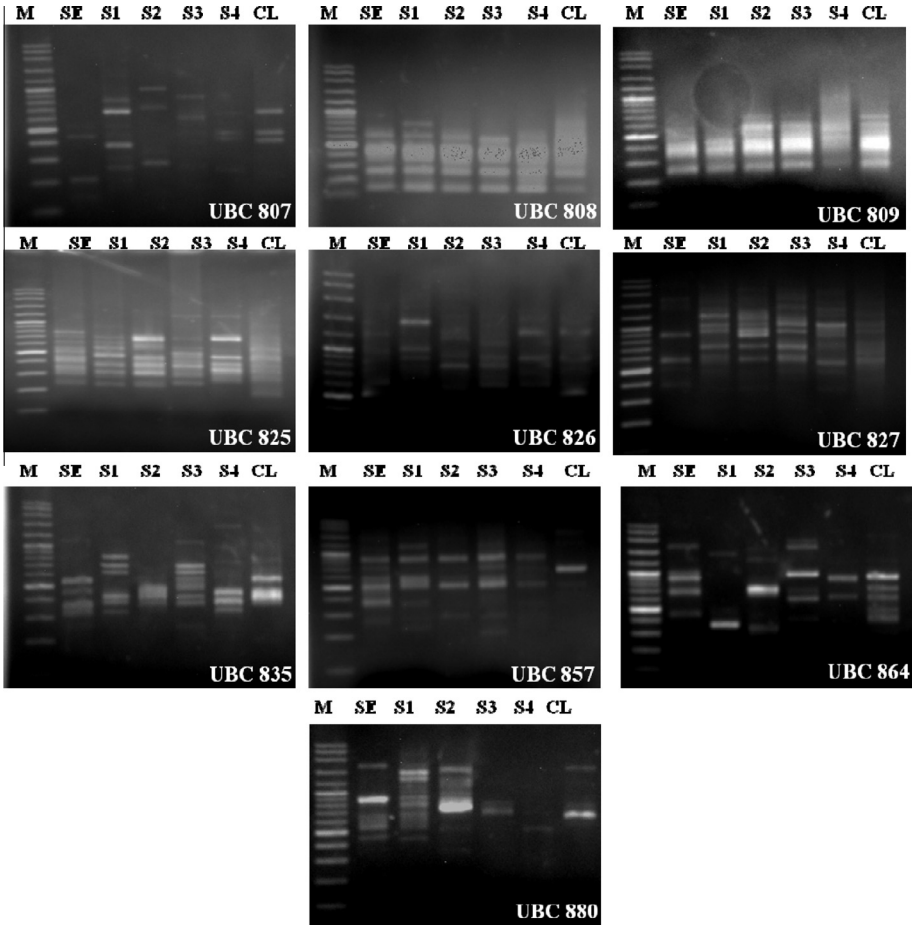

Figure 2 ISSR profiles of *Spirogyra* (M): 100 bp marker, (SE): *S. ellipsospora*, (S1): *Spirogyra* sp.1, (S2): *Spirogyra* sp.2, (S3): *Spirogyra* sp.3, (S4): *Spirogyra* sp.4 and (CL): *Cladophora* sp.

The species concept of *Spirogyra* is based on morphological characteristics, which are probably not accurately distinguishable in terms of classification, except by a specially trained individual (McCourt et al., 1986). Moreover, difficulties arise because they are small and soft and also have only a few stable morphological characteristics and are subject to phenotypic

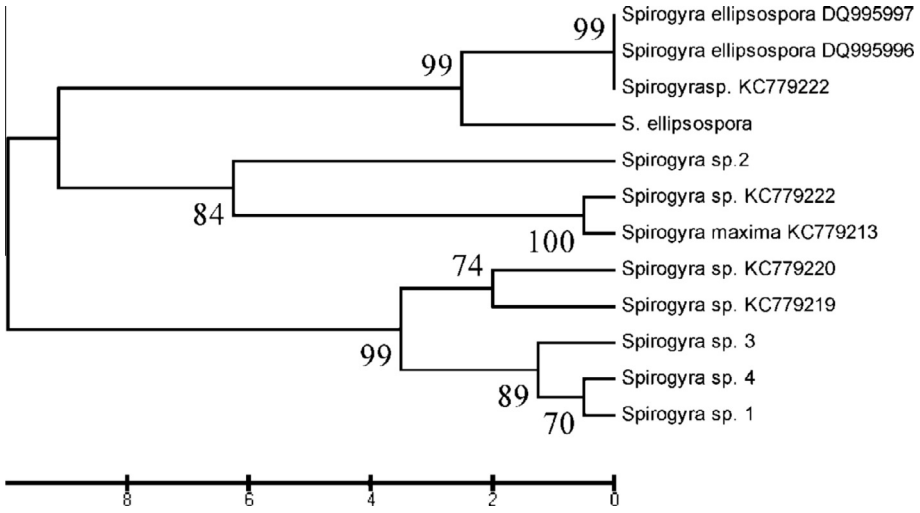

Figure 3 Phylogram derived from an UPGMA analysis depicting phylogenetic relationships of each morphological pattern of *Spirogyra* used in this study based on rbcL sequences.

variations. Thus, an identification of the closely related species of *Spirogyra* has only been based on morphological characteristics and as a result they can be confused or misidentified.

A previous study has considered the utility of the analysis of other organisms (Métais et al., 2000). Songdong (2008) screened ISSR primers to amplified green algae, *Chlorella vulgaris* genomic DNA and 18 primers were found to give reproducibly amplified products. When their results were compared with ours, ten ISSR primers (UBC 809, UBC 826, UBC 835, UBC 808, UBC 825, UBC 827, UBC 864, UBC 857, UBC 880 and UBC 807) were used for the investigation of the genetic diversity of the *Spirogyra* specimens. Moreover, all ISSR primers can be used for the molecular markers of deference of the *Spirogyra* species. Hence, the ISSR primers generated highly reproducible fragments and these were further used to study the genetic relationships of the *Spirogyra* populations of each region of Thailand.

The ten ISSR primers amplified a total of 92 fragments, varying from 5 to 12 fragments per primer and ranged from 100 to 2800 bp. An analysis of the ISSR markers separated the five *Spirogyra* specimens into two distinct clusters. This result corresponds to the cluster analysis of the rbcL gene, but with fewer differences in the sister clusters. The previous reports of Ratnaparkhe et al. (1995) reported an average of 8 markers per primer in *Cajanus cajan*. Maciel et al. (2001) reported on the generation of RAPD fragments ranging from 7 to 31 in common beans. Such a high variation in the number of fragments produced by these primers may be attributed to the differences in the binding site throughout the genome of the genotype. Ajibade et al. (2000) reported on the generation of the ISSR fragments ranging from 4 to 12 markers in *Vigna* and 8 markers in *Phaseolus vulgaris* (Galván et al., 2003). The distribution of the different microsatellite sequences in different living genomes determined the possibility of using this method for the purpose of DNA fingerprinting. This indicates that the ISSR marker is applicable in assessing molecular relativity among species of *Spirogyra*.

According to our phylogenetic analysis, the partial sequences of rbcL of each *Spirogyra* specimen are now known, which were previously poorly known in Thailand. The molecular methods using DNA sequencing technologies have been successfully developed for studying their phylogenetic relationships and the classifications of the unknown species of living organisms. Alignment of the rbcL sequence of *S. ellipsospora* 1, revealed it to be 99% identical with the *S. ellipsospora* data in the Genbank database, while *Spirogyra* sp.2 revealed that it was only 93% identical with *S. maxima*. This result is in contrast with the results of the morphological characteristics of this sample of *Spirogyra* sp.2 from this investigation, according to Thiamdao (2011). Stancheva et al. (2013) studied the *S. maxima* in California and reported the morphological characteristics of this species as follows: (1) cell 120–150 μm in width and 90–280 μm in long, (2) 5–8 chloroplasts per cell and (3) lenticular zygospores. In addition, because the rbcL data of *S. neglecta* was not available in the Genbank database, the % identical of this species was analyzed with maximum identities of "*S. maxima*". Therefore, from this study, new sequence data of rbcL of *Spirogyra* sp.2 was submitted to the NCBI databases.

An analysis of the rbcL gene confirmed the presence of five morphological patterns of *Spirogyra* in Thailand. The rbcL sequence obtained in this study confirmed the maximum identities compared with the sequence that was available in the Genbank databases. However, few genetic variations have been found among different patterns at the nucleotide level. Moreover, the individual *Spirogyra* clade found in this study is essentially the same as, and is well supported by, the bootstrap values. Drummond et al. (2005) indicated that *Spirogyra* is monophyletic, but still treated *Sirogonium* as a separate genus based on the rbcL data. They were unable to discover the morphological characteristics that were useful for a generic distinction, simply because the taxa are largely congruent (having a number of more or less loosely coiled chloroplasts, etc.). In addition, they also considered the shape and ornamentation of the chloroplast margin as a diagnostic feature, but our observations showed this characteristic to be variable and highly dependent on filament vitality. Other morphological characteristics, such as chloroplast number or cell width, are well known to be highly variable and could be related to polyploidy (Hoshow et al., 1987; Hoshaw and McCourt, 1998).

The UPGMA tree shows a group of multiples closely related to *Spirogyra*. The bootstrapping of the sequences indicates significant support for this group. Little genetic variations are ob-

served among different morphological traits at the nucleotide level. Since rbcL sequences are used in the study of the phylogenetic relationships of *Spirogyra*, they have been used in a number of other reported studies (Hamdam et al., 2013).

The sequence data of *rbcl* can be used to investigate the phylogenetic relationships of *Spirogyra*. The analysis invariably revealed a monophyletic tree for morphological triads. Each clade of the different patterns for each morphological triad was separated into sister groups that correlated with the morphological characteristics, such as cell length, cell width, number of chloroplast spirals, and number of granules.

The phylogenetic and systematic analysis of *Spirogyra* can be determined by a molecular approach using the sequencing of rbcL. We have established that species-level identifications can be achieved, and rbcL analysis actually provides a phylogenetic for these algae.

In conclusion, the phylogenetic and systematic identification of *Spirogyra* can be determined by a molecular approach using the sequence data of rbcL. We have established that species-level identifications can be achieved, and rbcL analysis actually provides the phylogenetic data for these algae.

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